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The ATM protein, encoded by the gene responsible for the human genetic disorder ataxia telangiectasia (A-T), is a 370 KDa protein with a COOH-terminal domain similar to the catalytic subunit of the phosphoinositide 3 kinases (PI 3-kinases). This places ATM within a family of protein kinases that function in maintenance of genome stability, in cell cycle control, and cellular responses to DNA damage. In order to address its proposed function in cell cycle regulation and DNA damage checkpoints, we propose to use the xenopus system for analysis of its functional role. We have cloned the Xenopus homologue of the human ATM gene and are in the process of analyzing its functional role. To this end we have successfully produced recombinant fragments of the ATM protein, including the kinase domain, in E. coli and by in vitro translation in reticulocyte lysates. In addition, we have raised polyclonal antibodies against recombinant protein made in E. coli. The antibody recognizes a single band of approximately 350 KDa on western blots from extracts of xenopus eggs and embryos. Finally, we have used these probes to follow the expression pattern of both mRNA and protein througout the cell cycle and during early development.

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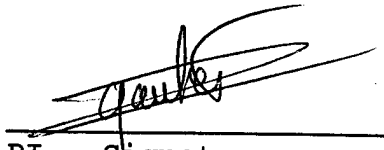

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THE FUNCTIONAL ROLE OF THE ATAXIATELANGIECTASIA GENE

ANNUAL REPORT - 09/98

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INTRODUCTION

Ataxia Telangiectasia is a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, neuronal degeneration, hypersensitivity to ionizing radiation, immunodeficiency and an increased cancer risk (Lavin and Shiloh, 1997). Epidemiological studies also point to a three to fourfold increased risk for breast cancer in A-T heterozygotes (Swift et al., 1991; Swift et al., 1987). The gene mutated in A-T, ATM, has been identified and encodes a 370 KDa protein that is a member of a protein kinase family (Savitsky et al., 1995; Savitsky et al., 1995). With knowledge of the ATM gene a number of studies have screened breast cancer patients for ATM mutations and these studies point towards an increased risk of breast cancer for A-T heterozygotes, but the size of the risk is still unknown (Lavin, 1998). An association between A-T heterozygosity and breast cancer was revealed by Athma et al. who concluded that 6.6% of all cases of breast cancer in America occur in AT-heterozygotes (Athma et al., 1996). In another study, an analysis of loss of heterozygosity (LOH) of ATM in sporadic breast cancer showed that 40% of cases analyzed showed a LOH for ATM, which correlated with a higher grade and a younger age at diagnosis in the set of tumors studied (Rio et al., 1998). However, another study concluded that ATM mutations do not confer genetic predisposition to breast cancer (FitzGerald et al., 1997). Clearly, large scale studies are required to address conclusively the association between mutations in ATM and risk of developing breast cancer.

ATM is a component of the cell cycle checkpoint machinery that causes growth arrest after ionizing radiation induced DNA damage (Lavin and Shiloh, 1997). Checkpoints monitor events during the cell cycle and coordinate their successful completion with subsequent transitions in the cycle (Hartwell and Weinert, 1989). Checkpoints which monitor the state of the DNA during the cell division cycle, negatively regulate cell cycle transitions when damage is detected, arresting cell cycle progression until such time as damage can be repaired (Murray, 1992).

Cell lines derived from A-T patients are hypersensitive to irradiation and show defects in several radiation induced cell cycle checkpoints (Kastan et al., 1992; Morgan and Kastan, 1997). Previous genetic and biochemical evidence implicated the ATM gene product in regulating the phosphorylation and induction of p53 in cells exposed to ionizing radiation. Most recent data indicate that ATM is a protein kinase whose activity is increased by ionizing radiation, and additionally was shown to directly phosphorylate p53 in response to DNA damage (Banin et al., 1998; Canman et al., 1998). This is consistent with the fact that ATM and p53 proteins have been shown to directly interact with each other (Watters et al., 1997). It has also been shown to interact with c-Abl as part of a radiation signal transduction pathway(s) (Baskaran et al., 1997; Shafman et al., 1997).

The eggs of the African clawed frog, *Xenopus laevis* provide a unique system for studying the cell cycle and are extensively used in the field of cell cycle research. It is possible to study cell division in intact embryos as well as in "cycling extracts" which faithfully mimic many aspects of the cell cycle *in vivo* (Murray and Kirschner, 1989). More recently, egg extracts have been used for the analysis of the role of the Chk1 protein kinase in the checkpoint response to unreplicated DNA (Kumagai et al., 1998).

In order to study the role of ATM in DNA damage checkpoints and cell cycle progression we have cloned the xenopus homologue of the ATM protein. Using the unique characteristics of the xenopus system we propose to study the role of ATM in cell cycle regulation and DNA damage checkpoint function. The proposed research will broaden our understanding of the biochemical function of the ATM protein.

BODY

This report describes the progress made in studying ATM for the award period, 1 September '97--31 August '98. During this first year our goal was to develop the tools to carry out a biochemical analysis of the xl ATM protein, particularly its role in cell cycle regulation and DNA damage checkpoints.

Xenopus laevis ATM

To date we have cloned an ≈5 kb fragment of the xl ATM cDNA by a combination of PCR amplification using degenerate oligonucleotides, library screening, and 5' RACE (Rapid Amplification of cDNA ends). The ATM gene is highly conserved between xenopus and human, and a schematic representation of the protein domains highlights the % identity between different regions of human and Xenopus ATM (Figure 1). Within the kinase domain a high degree of conservation was observed, with the xenopus and human sequences showing 85% identity. The high homology to the human protein is maintained in the central portion of the protein where the degree of identity was 66%.

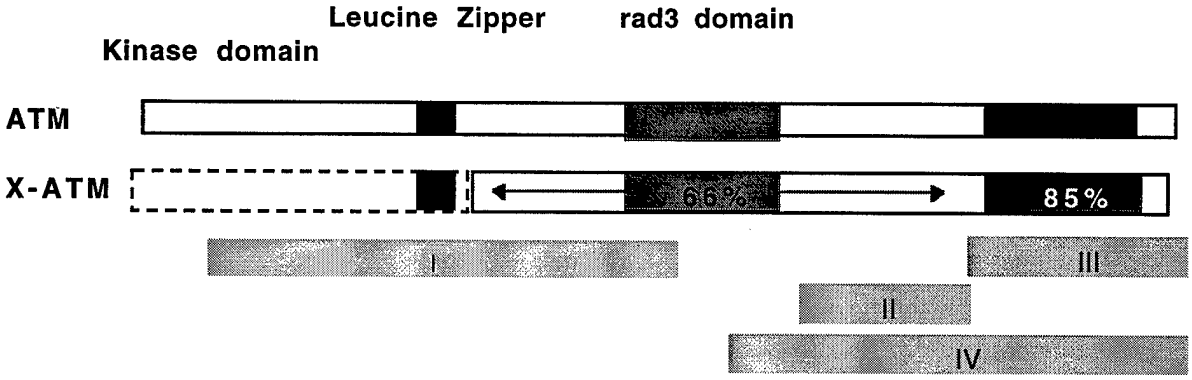

Figure 1. Schematic representation of the ATM proteins domains. The doted box for X-ATM represent the region of the cDNA that remains to be cloned. The gray boxes under the diagram represent the different truncated forms used for our studies. Box II is the region we used to generate antibodies against the X-ATM. Box III is the kinase domain region which we have constructed for both the human and Xenopus proteins. Box IV is the region we have cloned for expression in the baculovirus system. The numbers in the X-ATM boxes are the percent identities between Xenopus and human ATM proteins.

The expression of the ATM gene and purification of the protein represent important steps in an attempt to define the biochemical function of the protein. The focus in the past year has been recombinant protein and antibody

production, and the goals for the first 12 months, outlined below in specific aims 1 and 2, have largely been met.

Our technical objectives for the 4 years period of the grant were as follow:

1/ Expression of Xenopus ATM protein throughout the cell cycle. Studies in cell-free extracts Effect of overexpression of wild-type or mutant ATM on cell cycle progression and cell cycle checkpoint.

2/ Proteins binding to ATM: studies in cell-free extracts and two hybrid screen in yeast.

3/ Expression and functional studies of the ATM binding proteins, translation of the results to the breast epithelium model system.

More specifically the objective #1, spanning months 1-24 included:

A - Studies on ATM expression: months 1-12
B - Preparation of expression constructs for ATM wild-type and mutant: months 1- 12.
C - Expression of ATM in cell-free extracts: effect on cell cycle progression and on cell cycle checkpoints: months 12-24

Part A and B of objective #1 have been largely met, as we have completed the expression studies throughout the cell cycle and early development of Xenopus. This has been made possible because of the generation of very specific antisera against X-ATM, an extremely valuable tool that make us feel confident about the completion of the proposed research.

EXPERIMENTAL METHODS/ RESULTS

xl ATM cloning and protein expression.

A. Expression of a 21 KDa xl ATM fragment.

The ATM expression vector was constructed by sub-cloning a 480 bp fragment of the cloned cDNA into pQE-60 (Qiagen), (Figure 1, fragment II). The pQE expression vectors provide high level expression of proteins in E. coli, containing a 6 x His affinity tag allowing for isolation of the ATM protein by nickel chelate chromatography. A xl ATM fragment was isolated from pBSKSII-TBH4 using Nco1/BamH1 and cloned into these sites in pQE-60, where the coding sequence was under the control of the T7 polymerase promoter. The cloning junctions were sequenced to ensure the integrity of the sequence. The production of recombinant ATM was achieved by transforming the plasmid into M15 competent cells (Qiagen). Transformed E. coli were grown to log phase (A_{600}= 0.70), at 37°C, in the presence of ampicillin (50µg/ml) and kanamycin 25µg/ml. Cultures were induced by 2mM

isopropylthiogalactoside and time course experiments were undertaken to determine the optimal time for expression of the Xenopus ATM. It was determined that a 5 hour induction period was optimal for xl ATM expression.

B. Purification of xl ATM-21.

The recombinant *xl* ATM-21 was successfully purified using the hexahistidine tag at the N terminus of the protein. The Ni-NTA resin (Qiagen) was used to purify the His tagged xl ATM fragment form E. coli. Ni-NTA selectively binds proteins with six consecutive histidines; the 6xHis affinity tag. The expressed protein was found to be insoluble and was therefore purified under denaturing conditions using 6 M guanidine hydrochloride. The protein (4 mg/ml) was bound to the column in a batch procedure and eluted in 8 M Urea (Figure 2).

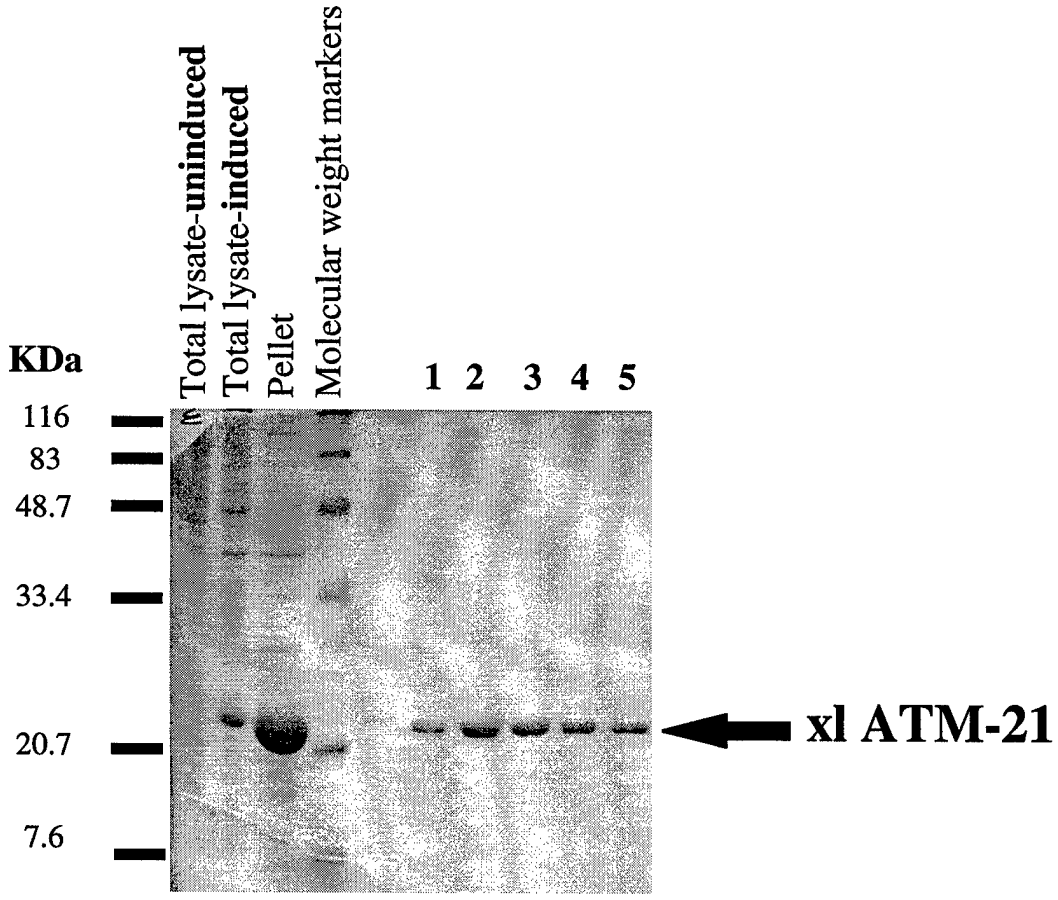

Figure 2. Expression of a fragment of xl ATM in E. coli. Analysis of ATM expression by SDS-PAGE (15%). Lanes 1 and 2 show the total cell lysate pre and post induction. Lanes 1-5 are fractions eluted from the Ni-chelate column.

Attempts to renature the protein by a stepwise dialysis in decreasing concentrations of urea were unsuccessful, this resulted in a total loss of the protein. Solubility was a general problem we experienced in attempts to express fragments of the xl ATM protein in E. coli. To improve recombinant protein production, and obtain soluble, functional protein, for example xl ATM kinase domain, we have decided to change our strategy for recombinant protein production and switch to the baculovirus expression system (see below).

B. Cloning and expression of the xl ATM kinase domain by in vitro transcription/translation.

A cDNA fragment including the complete kinase domain (Figure 1, III), was cloned into pBluescriptRN3, a vector for synthesizing in vitro transcribed mRNA. RNA was synthesized using mMessage mMachine (Ambion) and translated using a rabbit reticulocyte lysate translation system (Promega). Translation reactions using ^{35}S methionine yielded a protein product of the expected molecular weight upon analysis by SDS-PAGE (Figure 3).

We are thus ready to meet objective 3 (Months 12-18), that is the overexpression of the xl ATM in cleaving embryos, through RNA injections into one or two cell embryos. We will begin by injecting RNA encoding the entire PI 3-kinase domain since it has been reported that the ATM PI 3-kinase domain is catalytically active and can function to restore DNA damage induced cell cycle checkpoints in AT cells (Morgan et al., 1997).

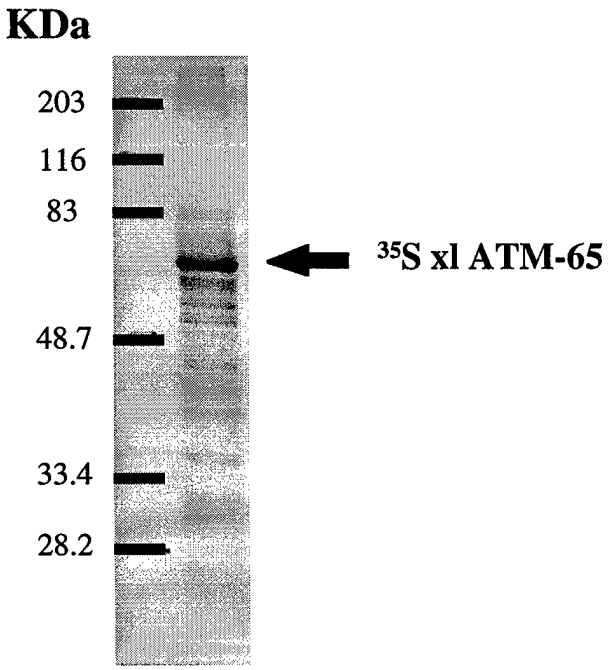

Figure 3. In vitro translation of the xl ATM kinase domain. Analysis of the in vitro translated ^{35}S labeled ATM by SDS-PAGE (10%). The ^{35}S xl ATM fragment migrated with a apparent molecular weight of 65 KDa.

C. Baculovirus expression

The baculovirus expression system provides an ideal system for overproducing recombinant proteins and their purification (Luckow 88; Miller, 88; Piwnica Worms 95). As a eukaryotic system it uses many of the protein modifications, processing, and transport systems present in higher eukaryotic cells. In addition, since the viral genome of the baculovirus is large, it can accommodate large segments of foreign DNA, such as the ATM cDNA. The successful expression and purification of full length recombinant human ATM has been reported using this system (Scott et al., 1998). In the past year we have established the baculovirus expression system and are successfully using it to express a number of xenopus cell cycle proteins. We are currently subcloning a large C-terminal xl ATM cDNA fragment (Figure1, IV) into a baculovirus expression vector for protein production in this system.

Polyclonal antibody production and purification.

A. Rabbit immunization

A professional antibody production service, Pocono Rabbit Farm and Laboratory Inc., was used. To prepare antigen for injection into rabbits xl ATM-21 was loaded on a preparative SDS-PAGE gel (15%). The ATM band was cut from the gel and electro-eluted into Tris/Glycine/SDS buffer. Electroeluted xl ATM-21, and acrylamide gel slices containing the protein, were provided as antigen. Two rabbits were maintained for a standard immunization protocol covering a period of six months.

B. Antisera testing

Antisera was supplied for testing on a weekly basis. Routine analysis was by SDS-PAGE followed by immunoblotting of the in vitro translated 65 KDa fragment of xl ATM.

Serum from both rabbits was found to cross react with both the 21 KDa ATM fragment used for immunization, and the larger in vitro translated fragment expressing the entire kinase domain (Figure 4).

Characterization of xl ATM antibodies

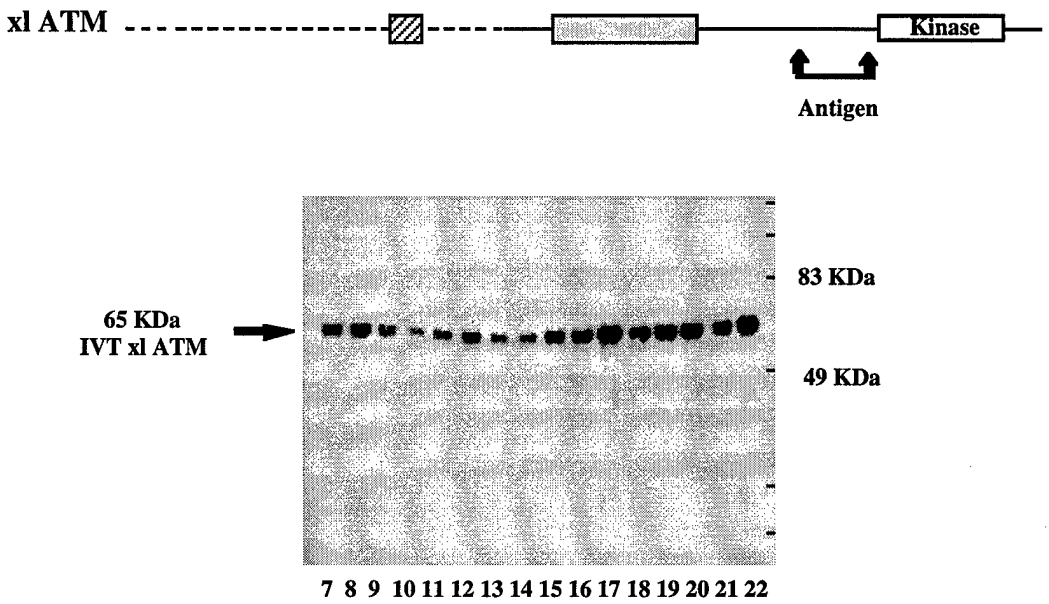

Figure 4. Analysis of xl ATM test bleeds. The region used to immunize rabbits is indicated in the schematic representation of the ATM protein domains. Weekly bleeds (no's 7-22), were immunoblotted against the in vitro translated xl ATM.

C. Antibody purification.

The crude serum recognized a single polypeptide of ≈350 Kda (Figure 5A, lane 1), which is close to the size of the human and mouse ATM protein and the predicted size of the xenopus protein. The antibody reaction was blocked by preincubation of the antiserum with the bacterially expressed ATM fragment (data not shown). The antibody was further purified by affinity chromatography using a column prepared by crosslinking the antigen to a CNBr activated sepharose. This yielded a highly specific IgG fraction that was used in subsequent experiments (Figure 5A, lane 2).

Analysis of Xenopus ATM using the α-ATM antibody

A. xl ATM forms high molecular weight complexes. Using non denaturing gradient polyacrylamide gels followed by western blotting we showed that the native xl ATM protein migrates as a monomer of about 350 KDa as well as a higher molecular weight complex of about 500 KDa (Figure 5B) The antibody can also precipitate a fraction of the ATM protein (Figure 5C). We will use this property to characterize the subunits of the high molecular weight ATM complex.

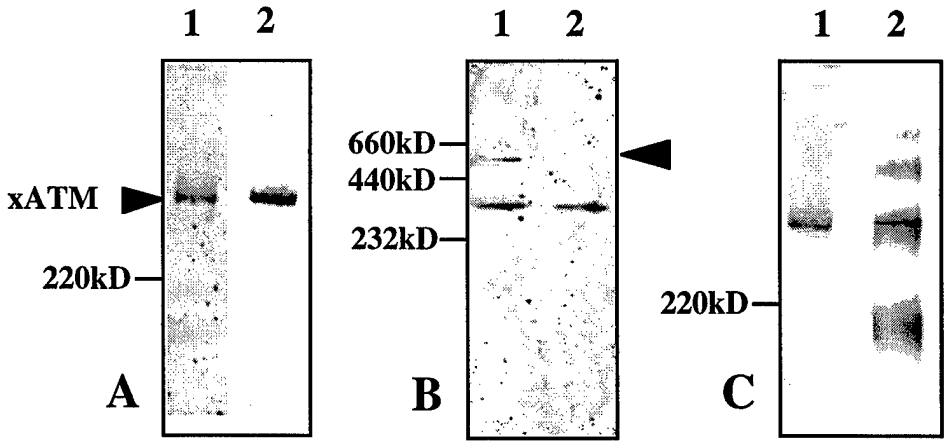

Figure 5. xl ATM antibodies recognize a ≈350 KDa polypeptide in xenopus egg extracts. (A) Specificity of the crude serum and the affinity purified antibody for xl ATM. 100 µg of Xenopus egg extract was electrophoresed (6 % SDS-PAGE) and western blotted with the crude serum (lane 1) or the affinity purified antibody (lane 2). **(B)** xl ATM forms high molecular weight complexes. 200 µg of extracts were electrophoresed under native conditions and immunoblotted with xl ATM antibody. A 500 KDa band (arrowhead) corresponding to the xenopus ATM complex is observed in addition to the 350 KDa monomeric xl ATM (lane 1). The complex is not a cross reactive band of high molecular weight as it shifts down when the sample is treated with SDS prior to electrophoresis (lane 2). **(C)** xl ATM antibody can immunoprecipitate xl ATM protein. 500 µg of extract were subjected to immunoprecipitation followed by western blotting using the xl ATM antibody (lane 2). A 350 KDa polypeptide of the same size as that in the control extract (lane 1) is immunoprecipitated.

B. Expression of xl ATM during the cell cycle. Since ATM has been proposed to be involved in the cell cycle checkpoint response following DNA damage it is important to assess whether ATM protein levels fluctuate throughout the cell cycle. Xenopus cycling extracts provide the ideal system for such an analysis as these extracts are undergoing genuine cell cycle transitions in a synchronous manner. We found that the level of xl ATM does not change throughout the cell cycle (Figure 6A).

C. Expression of Xenopus ATM during development. We have studied the temporal expression of xenopus ATM protein during early development. The protein is expressed maternally, and protein amount increases throughout the process of neurogenesis (Figure 6B).

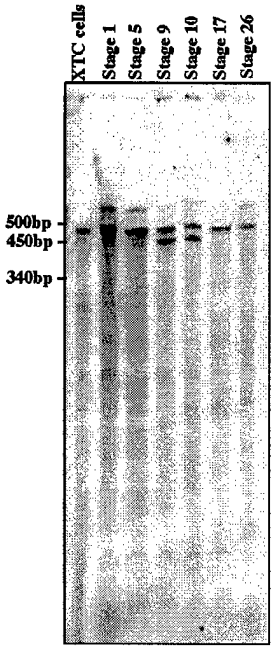

Figure 6: Expression of X-ATM mRNAs during early development. RNAse protection assays using a 480bp probes from the UTR of the X-ATM cDNA were performed using standard protocols at different stages of development and from RNA prepared from XTC cells (1st lane).

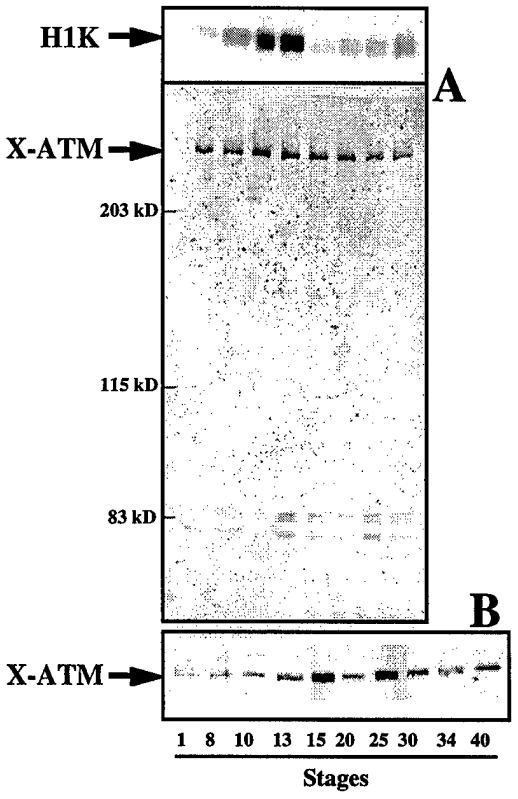

Figure 7. (A) Western blot showing the expression of ATM throughout development. The protein is detected from stage 1 and the level of expression increases through gastrulation (St. 13), and peaks at the time of formation of the neural tube and somites (St. 15-25), to slowly decrease thereafter.

CONCLUSIONS

We have successfully produced fragments of the Xenopus ATM protein by various recombinant technologies. We are continuing to work on expression of functionally active ATM domains using the baculovirus system. In addition, we were successful in raising antibodies specific for the Xenopus ATM protein, and these antibodies identified xenopus ATM as a 350 KDa protein in xenopus egg extracts. Using the antibody we were able to determine that ATM is part of a large molecular weight protein complex and remains at a constant level throughout the cell cycle.

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